Proteomic Landscape of Aldosterone-Producing Adenoma

Swierczynska, Marta M ; Betz, Matthias J ; Colombi, Marco ; Dazert, Eva ; Jenö, Paul ; Moes, Suzette ; Pfaff, Cécile ; Glatz, Katharina ; Reincke, Martin ; Beuschlein, Felix ; Donath, Marc Y ; Hall, Michael N

Abstract: Primary aldosteronism is a disease of excessive production of adrenal steroid hormones and the most common cause of endocrine hypertension. Primary aldosteronism results mainly from bilateral adrenal hyperplasia or unilateral aldosterone-producing adenoma (APA). Primary aldosteronism cause at the molecular level is incompletely understood and a targeted treatment preventing excessive adrenal steroid production is not available. Here, we perform deep quantitative proteomic and phosphoproteomic profiling of 6 pairs of APA and adjacent nontumoral adrenal cortex. We show that increased steroidogenesis in APA is accompanied by upregulation of steroidogenic enzymes (HSD3B2, CYP21A2, CYP11B2) and of proteins involved in cholesterol uptake (LSR). We demonstrate that HSD3B2 is phosphorylated at Ser95 or 96 and identify a novel phosphorylation site, Ser489, in CYP21A2, suggesting that steroidogenic enzymes are regulated by phosphorylation. Our analysis also reveals altered ECM (extracellular matrix) composition in APA that affects ECM-cell surface interactions and actin cytoskeleton rearrangements. We show that RHOC, a GTPase controlling actin organization in response to extracellular stimuli, is upregulated in APA and promotes expression of the aldosterone synthase gene CYP11B2. Our data also indicate deregulation of protein N-glycosylation and GABAergic signaling in APAs. Finally, we find that mTORC1 (mammalian target of rapamycin complex 1) signaling is the major pathway deregulated in APA. Our study provides a rich resource for future research on the molecular mechanisms of primary aldosteronism.

DOI: https://doi.org/10.1161/HYPERTENSIONAHA.118.11733

Proteomic Landscape of Aldosterone-Producing Adenoma

Marta M. Swierczynska, Matthias J. Betz, Marco Colombi, Eva Dazert, Paul Jenö, Suzette Moes, Cécile Pfaff, Katharina Glatz, Martin Reincke, Felix Beuschlein, Marc Y. Donath, Michael N. Hall

See Editorial Commentary, pp 284–285

Abstract—Primary aldosteronism is a disease of excessive production of adrenal steroid hormones and the most common cause of endocrine hypertension. Primary aldosteronism results mainly from bilateral adrenal hyperplasia or unilateral aldosterone-producing adenoma (APA). Primary aldosteronism cause at the molecular level is incompletely understood and a targeted treatment preventing excessive adrenal steroid production is not available. Here, we perform deep quantitative proteomic and phosphoproteomic profiling of 6 pairs of APA and adjacent nontumoral adrenal cortex. We show that increased steroidogenesis in APA is accompanied by upregulation of steroidogenic enzymes (HSD3B2, CYP21A2, CYP11B2) and of proteins involved in cholesterol uptake (LSR). We demonstrate that HSD3B2 is phosphorylated at Ser95 or 96 and identify a novel phosphorylation site, Ser489, in CYP21A2, suggesting that steroidogenic enzymes are regulated by phosphorylation. Our analysis also reveals altered ECM (extracellular matrix) composition in APA that affects ECM-cell surface interactions and actin cytoskeleton rearrangements. We show that RHOC, a GTPase controlling actin organization in response to extracellular stimuli, is upregulated in APA and promotes expression of the aldosterone synthase gene *CYP11B2*. Our data also indicate deregulation of protein N-glycosylation and GABAergic signaling in APAs. Finally, we find that mTORC1 (mammalian target of rapamycin complex 1) signaling is the major pathway deregulated in APA. Our study provides a rich resource for future research on the molecular mechanisms of primary aldosteronism. (***Hypertension*. 2019;73:469-480. DOI: 10.1161/HYPERTENSIONAHA.118.11733.)**
● **Online Data Supplement**

Key Words: adenoma ■ human ■ hyperaldosteronism ■ hypertension ■ mass spectrometry

Primary aldosteronism (PA) is the most common form of endocrine hypertension, affecting 6% of the general hypertensive population and 11% of patients in specialized hypertension referral centers.[1] PA most commonly results from bilateral adrenal hyperplasia (BAH) and unilateral aldosterone-producing adenoma (APA), which together account for >95% of PA cases.[2] Independent of hypertension, PA is associated with an increased risk of hypokalemia, cardiovascular and metabolic complications, renal impairment, osteoporosis, depression, and anxiety.[3,4] These conditions are attributable to elevated production of aldosterone, glucocorticoids, and adrenal androgens.[3] Current PA treatment includes adrenalectomy for APA patients and mineralocorticoid receptor antagonists for BAH, as well as APA patients who are not eligible for surgery.[2] Pharmacological inhibition of mineralocorticoid receptors normalizes blood pressure and hypokalemia but does not address metabolic complications and osteoporosis.[4] Thus, additional therapeutic strategies are needed.

Approximately 50% of APAs harbor somatic mutations in genes encoding ion channels or pumps (*KCNJ5*, *CACNA1D*, *ATP1A1*, *ATP2B3*) that regulate intracellular ion homeostasis and cell membrane potential.[5] Other molecular events leading to PA development are still incompletely understood. Transcriptome profiling has identified genes with a potential role in APA pathophysiology.[5] However, biological processes are driven by proteins and transcript levels often do not predict protein amounts.[6] Mass spectrometry allows proteomic analysis including the identification of posttranslational modification in an unbiased, quantitative, and sensitive manner.[6] Here, we use this powerful method to profile the proteome and phosphoproteome of APA and matched nontumoral adrenal cortex. In addition to confirming disturbances characterized in previous studies, we identify novel pathways potentially involved in APA development and its hormonal autonomy. Our observations provide insight into APA pathophysiology that may translate into novel PA treatments.

Received June 27, 2018; first decision July 12, 2018; revision accepted October 20, 2018.

From the Biozentrum, University of Basel, Switzerland (M.M.S., M.C., E.D., P.J., S.M., C.P., M.N.H.); Clinic of Endocrinology, Diabetes & Metabolism (M.J.B., M.Y.D.), and Institute of Pathology (K.G.), University Hospital Basel, Switzerland; Medizinische Klinik und Poliklinik IV, Klinikum der Universität München, Munich, Germany (M.R., F.B.); and Klinik für Endokrinologie, Diabetologie und Klinische Ernährung, UniversitätsSpital Zürich, Switzerland (F.B.).

The online-only Data Supplement is available with this article at https://www.ahajournals.org/doi/suppl/10.1161/HYPERTENSIONAHA.118.11733.

Correspondence to Michael N. Hall, Klingelbergstrasse 70, CH-4056 Basel, Switzerland. Email m.hall@unibas.ch

Methods

The authors declare that all supporting data are available within the article and its online-only Data Supplement.

Subjects

Adrenal tissue was collected from patients undergoing adrenalectomy for APA at the University Hospital Munich. PA/APA diagnosis was in accordance with institutional guidelines. Postoperative histological examination confirmed APA in all patients except patient 3 in which 3 distinct (6,7, and 10 mm) macronodules were identified. It was not determined whether the macronodules were because of nodular hyperplasia or were 3 independent adenomas. For all molecular analyses of patient 3, only material from the dominant 10 mm nodule was taken. Given the histopathologic findings, all tumoral samples are hereafter referred to as APAs or adenomas. In all samples, APA-associated genes (*KCNJ5*, *ATP1A1*, *ATP2B3, CACNA1D*, *CACNA1H*, *CTNNB1*) and *PRKACA* were sequenced, as described in another study.[7] Sequencing results and patients' clinical characteristics are provided in Table S1 in the online-only Data Supplement. All patients provided written informed consent. The study was approved by the Ethics Committee of the Ludwig-Maximilian University of Munich.

For detailed description of materials and methods see Materials and Methods in the online-only Data Supplement.

Results

Quantitative Proteomics and Phosphoproteomics of APA

We compared the proteomes and phosphoproteomes, hereafter collectively referred to as (phospho)proteomes, of 6 pairs of APA and matched nontumoral adrenal cortex (Ctrl) (Figure 1A). The matched nontumoral tissue contained cells of all 3 zones of the adrenal cortex. On average, our deep quantitative mass spectrometry quantified around 7000 proteins per patient, of which 12% to 28% were deregulated in APAs (Figure 1B). Proteins were considered deregulated when they were differentially expressed by >0.585 log2 fold (>1.5-fold; $P<0.05$) or they were detected exclusively in Ctrl or APA samples. We detected and quantified 5555 proteins common to all samples, of which 18 were significantly downregulated and 11 were significantly upregulated in all APAs (Figure 1B; Tables S4 and S5). Depending on the amount of the input material, we quantified between 1036 and 4075 phosphosites per patient, which corresponded to 724–1969 phosphoproteins (Figure 1C and 1D; Table S2). Up to 50% of quantified phosphoproteins had at least 1 phosphosite that was significantly deregulated in APA (Figure 1D). We were able to quantify 870 specific phosphosites in 785 phosphoproteins in at least 4 patients, but there was no phosphosite common to all samples (Figure 1C and 1D).

Steroidogenesis in APA Is Deregulated at Many Levels

Steroid hormones are synthesized from cholesterol in a series of reactions catalyzed by mitochondria- and ER-associated enzymes (Figure 2A). We found that in addition to an expected increase in aldosterone synthase (CYP11B2) expression, APAs showed higher levels of HSD3B2 and CYP21A2, the enzymes common to all steroid synthesis pathways (Figure 2A and 2B; Figure S1). Levels of enzymes specifically involved in glucocorticoid and androgen synthesis were either not affected (CYP17A1, CYP11B1) or only modestly downregulated (AKR1C3) in APAs (Figure 2B; Figure S1). Expression of STAR, a protein controlling cholesterol transport across the inner mitochondrial membrane, was not changed in APAs (Figure 2B; Figure S1). The increase in expression of steroidogenic enzymes observed in the APA samples most likely contributes to the higher aldosterone synthesis characteristic of PA. The enhanced expression of HSD3B2 and CYP21A2 could account for increased levels of circulating glucocorticoids and androgens as observed in a significant portion of PA patients.

Previous studies have reported differential expression of steroidogenic enzymes in APAs harboring the KCNJ5 mutation (hereafter referred to as $KCNJ5^{mut}$).[8–10] We observed lower levels of glucocorticoid and androgen producing enzymes (CYP11B1, CYP17A1, AKR1C3) in $KCNJ5^{mut}$ compared with $KCNJ5^{WT}$ adenomas, while aldosterone synthase levels showed the opposite trend (Figure S2). CYP11A1, HSD3B2, and CYP21A2 were unchanged (Figure S2). Furthermore, expression of steroidogenic enzymes was comparable in all control tissues (Figure S2). Our observed changes in expression of CYP11B2/B1/17 are consistent with that reported in previous transcriptomic studies.[9,10] However, they differ from those reported in an immunohistochemistry study which showed lower expression of CYP11B2 and higher expression of CYP17A1 in $KCNJ5^{mut}$ adenomas.[8] Whether this apparent discrepancy is because of experimental differences or other factors is unknown.

Steroidogenic output is influenced not only by the absolute levels of steroidogenic enzymes but also by their specific activity. It is known that the activity of STAR and CYP17A1 is regulated by a cAMP-dependent phosphorylation of Ser195[11] and an undetermined Ser/Thr site,[12] respectively. STAR phosphorylation was not affected in APAs (Figure S3), suggesting that this modification does not contribute to increased steroid hormone production. However, our phosphoproteomic analysis identified 2 new phosphorylation sites in other steroidogenic enzymes, Ser95 or 96 (Ser95/96) in HSD3B2 and Ser489 in CYP21A2. HSD3B2-pSer95/96, a predicted PKA target (www.cbs.dtu.dk/services/NetPhos), was quantified and found to be deregulated in 3 APAs (Figure 2C). CYP21A2-pSer489, predicted to be phosphorylated by GSK3 and other unspecified kinases, was quantified in 2 APAs and found to be upregulated in 1 (Figure 2C). These observations suggest that protein kinases regulate steroidogenic enzyme activity.

Oxidation reactions catalyzed by mitochondrial steroidogenic enzymes require FDX1 and FDXR for electron transfer, whereas steroidogenic enzymes of the ER require POR and CYB5. APAs displayed a decrease in FDX1 and CYB5A expression and normal FDXR and POR levels (Figure 2D; Figure S1). Thus, elevated steroidogenic output in APA seems not to be driven by increased electron transfer. It is not clear whether decreased FDX1 and CYB5A levels could negatively impact steroidogenic output and thereby constitute a mechanism to counter increased expression of steroidogenic enzymes. This requires further study.

Excessive steroidogenesis requires increased cholesterol supply. This can be achieved by upregulating either de novo synthesis, mobilization of cholesterol from intracellular

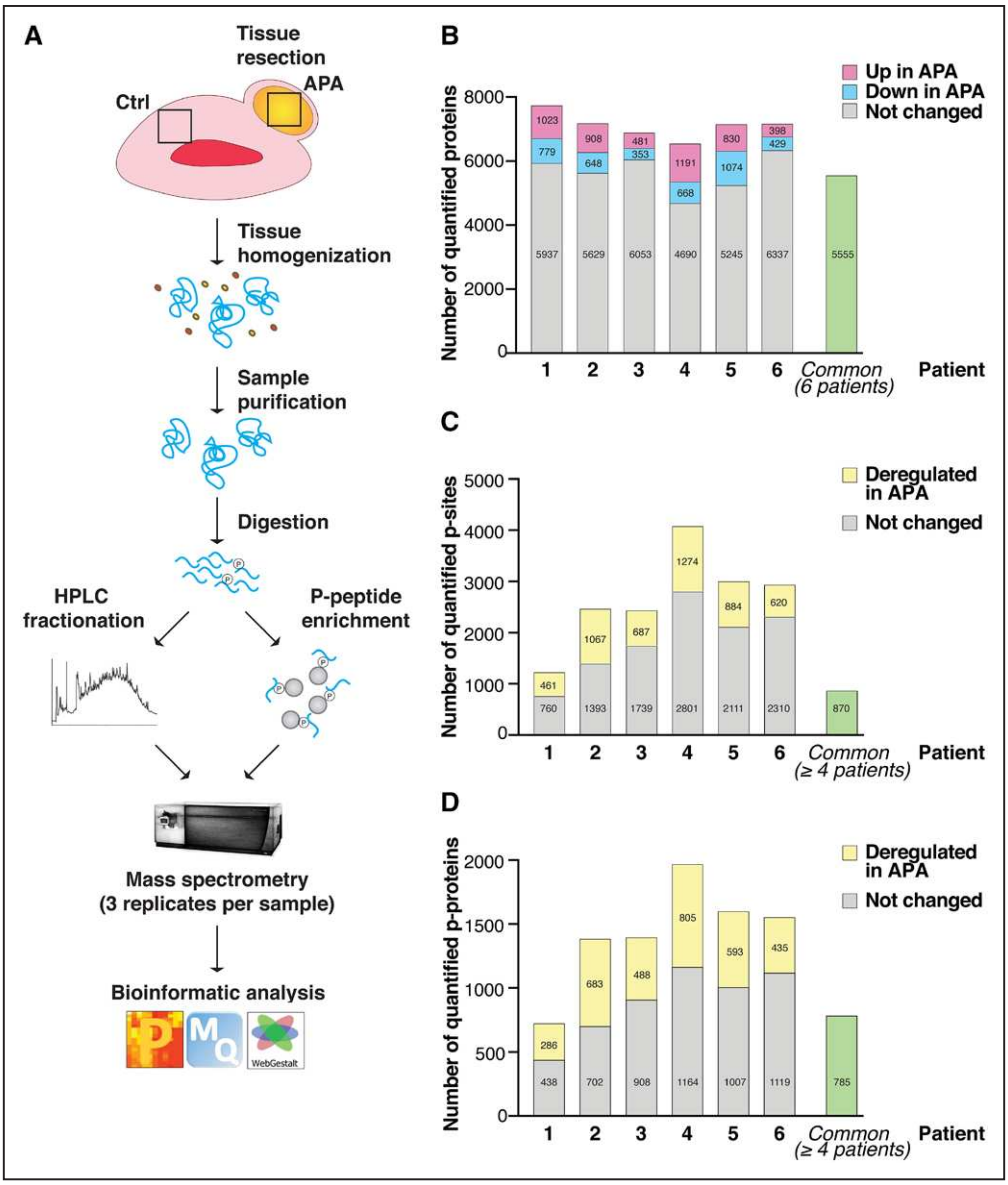

Figure 1. Experimental setup for deep quantitative aldosterone-producing adenoma (APA) (phospho)proteomics. **A**, Schematic overview of the study. **B**, Proteins, (**C**) phosphosites (p-sites), and (**D**) phosphoproteins (p-proteins) quantified and deregulated in APAs of individual patients.

lipid droplets, or lipoprotein uptake.[13] De novo cholesterol synthesis most likely does not contribute to increased steroidogenesis since neither of the quantified enzymes involved in this pathway was upregulated in APAs (Figure 2E). Mobilization of cholesterol from lipid droplets is also an unlikely contributor—HSL (an enzyme mobilizing cholesterol from lipid droplets) and SOAT1 (an enzyme modulating lipid droplets assembly) were downregulated and upregulated, respectively, in APAs (Figure 2E; Figure S1). Surprisingly, canonical lipoprotein receptors were either unchanged (VLDLR, LDLR, SR-B1) or downregulated (CD36) in APAs (Figure 2E; Figure S1). Likewise, proteins mobilizing lipoprotein-derived cholesterol were not changed (NPC1 [Niemann-Pick C1], LIPA) or showed only a modest increase (NPC2; Figure 2E; Figure S1). Among the lipoprotein receptors, lipolysis-stimulated lipoprotein receptor (LSR), which binds VLDL and LDL, was upregulated in adenomas (Figure 2E; Figure S1). Thus, it can be speculated that cholesterol required to support increased steroidogenesis in APAs comes from LDL and is taken up via LSR.

Clusters of Downregulated and Upregulated Proteins in APA

To visualize the variance of the proteomic data, we performed principal component analysis (PCA) of proteins detected in all mass spectrometry runs of all patient (5077 proteins in total). This analysis revealed that adenoma proteomes differ from those of control adrenal cortex (Figure 3A). In addition, PCA showed that APA samples harboring KCNJ5^{mut} are distinct from those with the wild-type version of the channel (Figure 3A). Interestingly, also Ctrl samples corresponding to KCNJ5^{mut} adenomas clustered and were clearly separated from other control samples (Figure 3A). This may suggest that

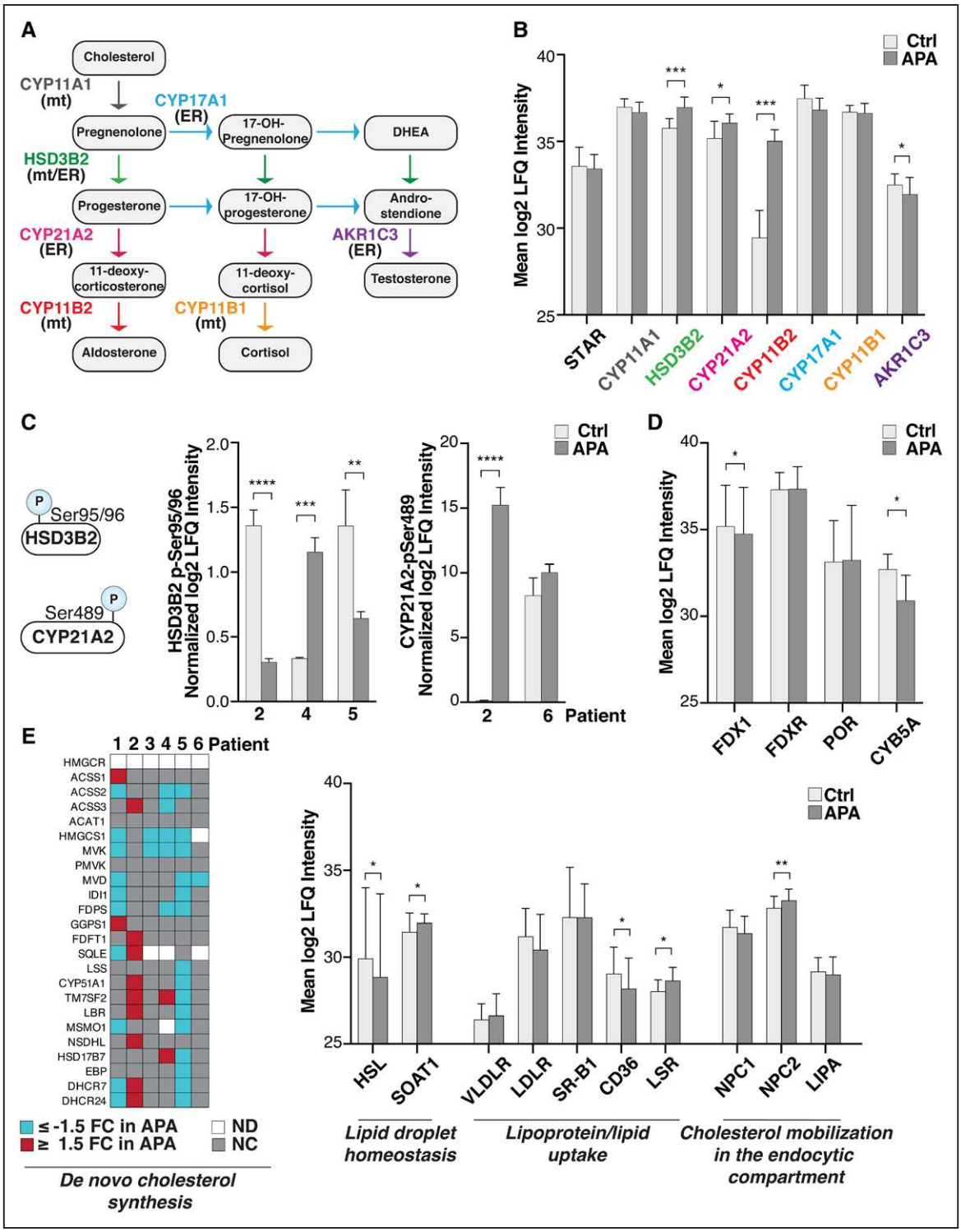

Figure 2. Steroidogenesis in aldosterone-producing adenoma (APA) is deregulated at multiple levels. **A**, Overview of adrenal steroidogenic pathways. (mt): mitochondria-associated enzymes, (ER): ER-associated enzymes. **B**, Expression of steroidogenic enzymes in APA. **C**, Quantification of HSD3B2 and CYP11B2 phosphorylation on newly discovered sites. **D**, Expression of enzymes involved in electron transport to the steroidogenic enzymes in APA. **E**, Expression of proteins involved in cholesterol synthesis and trafficking in APA. ND: Not detected. NC: Not changed. **B**–**E**, Data are presented as means±SD.*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 (paired t test).

KCNJ5^{mut} affects not only the tumor but the whole gland, for example, by promoting secretion of a paracrine factor or by inducing a systemic response affecting other adrenocortical cells. In summary, PCA demonstrates that APAs share many characteristics at the proteome level and clearly differ from the surrounding nontumoral adrenal cortex, irrespective of their genetic background.

To identify proteins with a similar expression pattern across all APAs, we performed unsupervised hierarchical clustering of proteins that were quantified in all samples and

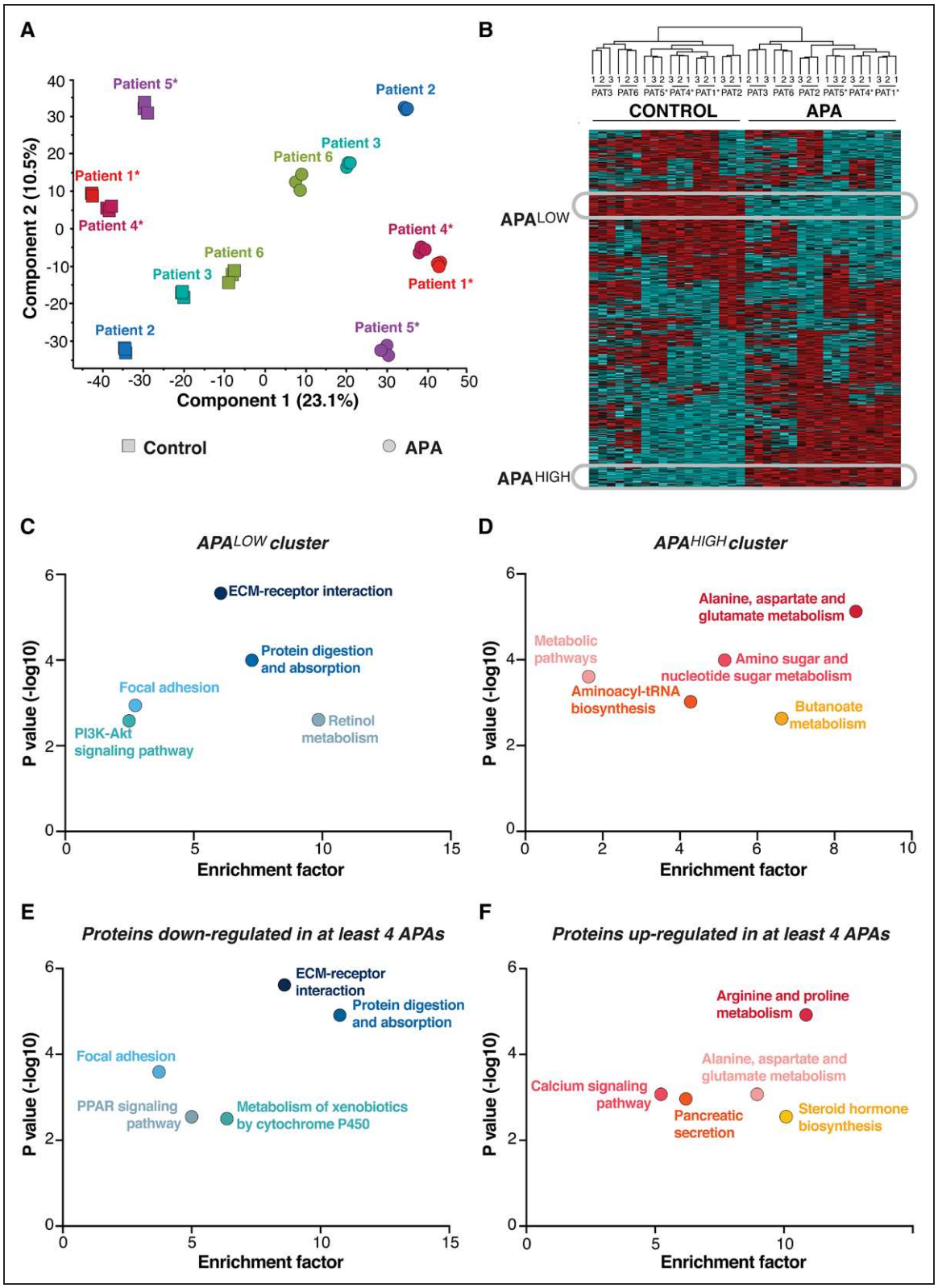

Figure 3. Proteomic analysis reveals proteins differentially regulated in aldosterone-producing adenoma (APA). **A**, Principal component analysis of proteins detected in each mass spectrometry measurement of all the samples. * denotes patients harboring KCNJ5^{mut}. **B**, Unsupervised hierarchical clustering of proteins quantified in all samples and deregulated in at least 2 adenomas. **C** and **D**, KEGG pathway enrichment for proteins of (**C**) APA^{LOW} and (**D**) APA^{HIGH} clusters. **E** and **F**, KEGG pathway enrichment for proteins (**E**) downregulated and (**F**) upregulated in at least 4 adenomas.

significantly deregulated in at least 2 adenomas (3210 proteins in total). Clustering was based on the normalized expression data (*Z* scores). Column-wise clustering confirmed the PCA results, demonstrating that APA proteomes differ from those of the Ctrl adrenal cortex, and that samples from patients harboring KCNJ5^{mut} cluster (Figure 3B). Row-wise clustering

revealed the presence of 2 prominent clusters of proteins with a similar expression pattern in APAs (Figure 3B). These clusters, termed APA^{LOW} and APA^{HIGH}, contain proteins downregulated and upregulated in APA, respectively (Tables S6 and S7).

We performed KEGG and Reactome pathway enrichment analyses of proteins within the APA^{LOW} and APA^{HIGH} clusters. The APA^{LOW} cluster was particularly enriched in pathway terms related to ECM (extracellular matrix) homeostasis and ECM-cell interactions (Figure 3C; Figure S4). In contrast, the APA^{HIGH} cluster was particularly enriched in pathway terms related to protein N-linked glycosylation and amino acid metabolism (Figure 3D; Figure S4). Consistent with the characteristics of adenomas, the APA^{HIGH} cluster was also enriched in proteins involved in steroidogenesis (Figure S4).

To validate the results of the *Z* score-based hierarchical clustering, we manually searched for proteins significantly dowregulated or upregulated in at least 4 APAs (Tables S8 and S9) and performed pathway enrichment analyses of these 2 protein groups. Consistent with previous results, commonly downregulated proteins corresponded to pathways involved in ECM homeostasis and ECM-receptor interactions (Figure 3E; Figure S5), while upregulated proteins corresponded to amino acid metabolism and steroidogenesis (Figure 3F; Figure S5). In addition, commonly upregulated proteins were linked to calcium signaling (Figure 3F; Figure S5).

PCA and unsupervised hierarchical clustering suggested that $KCNJ5^{mut}$ APAs are more similar to each other than to $KCNJ5^{WT}$ adenomas. This prompted us to search manually for proteins whose expression is altered in all 3 $KCNJ5^{mut}$ APAs. Out of 142 proteins commonly downregulated in $KCNJ5^{mut}$ tumors, 57 showed reduced expression exclusively in the $KCNJ5^{mut}$ cohort while 85 were also downregulated in at least one $KCNJ5^{WT}$ adenoma (Tables S10 and S11). Likewise, 89 of 173 proteins commonly upregulated in $KCNJ5^{mut}$ tumors showed higher expression only in these tumors (Tables S12 and S13). Pathway enrichment analyses revealed that proteins downregulated specifically in $KCNJ5^{mut}$ APAs are involved mainly in xenobiotic metabolism, whereas the upregulated proteins are involved in ion transport across plasma membrane and iron homeostasis (Figure S6). These findings imply that, while sharing many characteristics with the $KCNJ5^{WT}$ tumors, $KCNJ5^{mut}$ adenomas display a distinct protein expression pattern.

To complement our study, we performed pathway enrichment analyses of proteins downregulated or upregulated in APAs of individual patients and created a ranking of commonly deregulated pathways among all patients. This analysis confirmed that pathways commonly downregulated in APAs were related to maintaining ECM homeostasis and ECM-cell interactions, whereas the commonly upregulated pathways corresponded to N-glycosylation and calcium signaling (Figure S7). In addition, this analysis identified oxidative phosphorylation to be commonly upregulated in adenomas (Figure S7).

APA Is Characterized by Disturbed ECM-Cell Surface-Cytoskeleton Interactions

All the above analyses indicate downregulation of pathways related to the ECM and ECM-cell interactions in APA (Figure 4A). Most adenomas showed significantly lower content (>1.5-fold, $P<0.05$) of various collagen types (COL1A1-2, COL6A1-3, COL14A1), proteins mediating collagen fibril assembly (COL14A1, FMOD, LUM, BGN, DCN, PCOLCE, ASPN), other ECM structural components (EFEMP2, PRELP, DPT, LAMC3), and enzymes involved in ECM turnover (PCOLCE, CMA1, TPSAB1). Likewise, expression of ITG7A (integrin α-7), which provides the link between the ECM and intracellular signaling, was downregulated in all adenomas tested (Table S4). The actin cytoskeleton, which regulates various cellular processes in response to the extracellular signals,[14] also appeared to be affected in APA—a few cytoskeleton-associated proteins (MYLK, FLNC) displayed reduced expression in adenomas compared with the matched adrenal cortex. In contrast, RHOC, a GTPase activated in response to extracellular stimuli and controlling formation and organization of the actin cytoskeleton,[15] was upregulated in all APAs (Table S5). Thus, APAs showed disturbed ECM-cell interactions at all possible levels: ECM composition, cell surface receptor expression, and actin cytoskeleton rearrangements (Figure 4A).

Protein N-Linked Glycosylation Is Increased in APA

Pathway enrichment analyses of proteins with increased expression in APAs indicated that N-linked glycosylation is upregulated in adenomas. N-glycosylation entails transfer of preassembled oligosaccharide from dolichol phosphate to an asparagine residue in a process catalyzed by a heterooligomeric enzyme oligosaccharyltransferase (OST)[16](Figure 4B). Multiple enzymes involved in the oligosaccharide assembly were significantly ($P<0.05$) upregulated >1.5-fold in 3 (ALG2, ALG6, ALG14) or 2 (ALG1, ALG11) adenomas. In addition, APAs displayed increased content of the OST complex components when compared with the corresponding control adrenal cortex. In particular, RNP1, RNP2, DDOST, STT3A, and MAGT1 were significantly upregulated at least 1.45-fold in 3 adenomas, and expression of DAD1 and OSTC was significantly increased >1.5-fold in 2 APAs. Finally, TUSC3, an accessory component of the OST complex, was > 2-fold upregulated in 4 APAs. Adenomas also displayed higher content of enzymes participating in formation of complex N-glycans, including MGAT2 (>1.5-fold increase in 3 adenomas) and MGAT1 and MAN1A2 (>1.5-fold increase in 2 APAs). In summary, APAs showed increased expression of enzymes involved in N-glycan assembly and thereby most likely higher rates of protein N-linked glycosylation (Figure 4B). Of note, N-linked glycosylation was one of the top upregulated pathways in patients 1 through 4 (Figure S5) and was therefore not related to the KCNJ5 status.

GABA Degradation Is Enhanced in APA

In the performed KEGG pathway enrichment analyses, proteins upregulated in APA corresponded to pathways related to amino acid (KEGG terms Alanine, aspartate and glutamate metabolism and Arginine and proline metabolism) and butanoate metabolism (Figure 3D and 3F; Figures S4 and S5). Most proteins we detected linked to these terms are involved in various independent pathways. Only 2 are involved in the same pathway, namely GABA degradation. GABA,

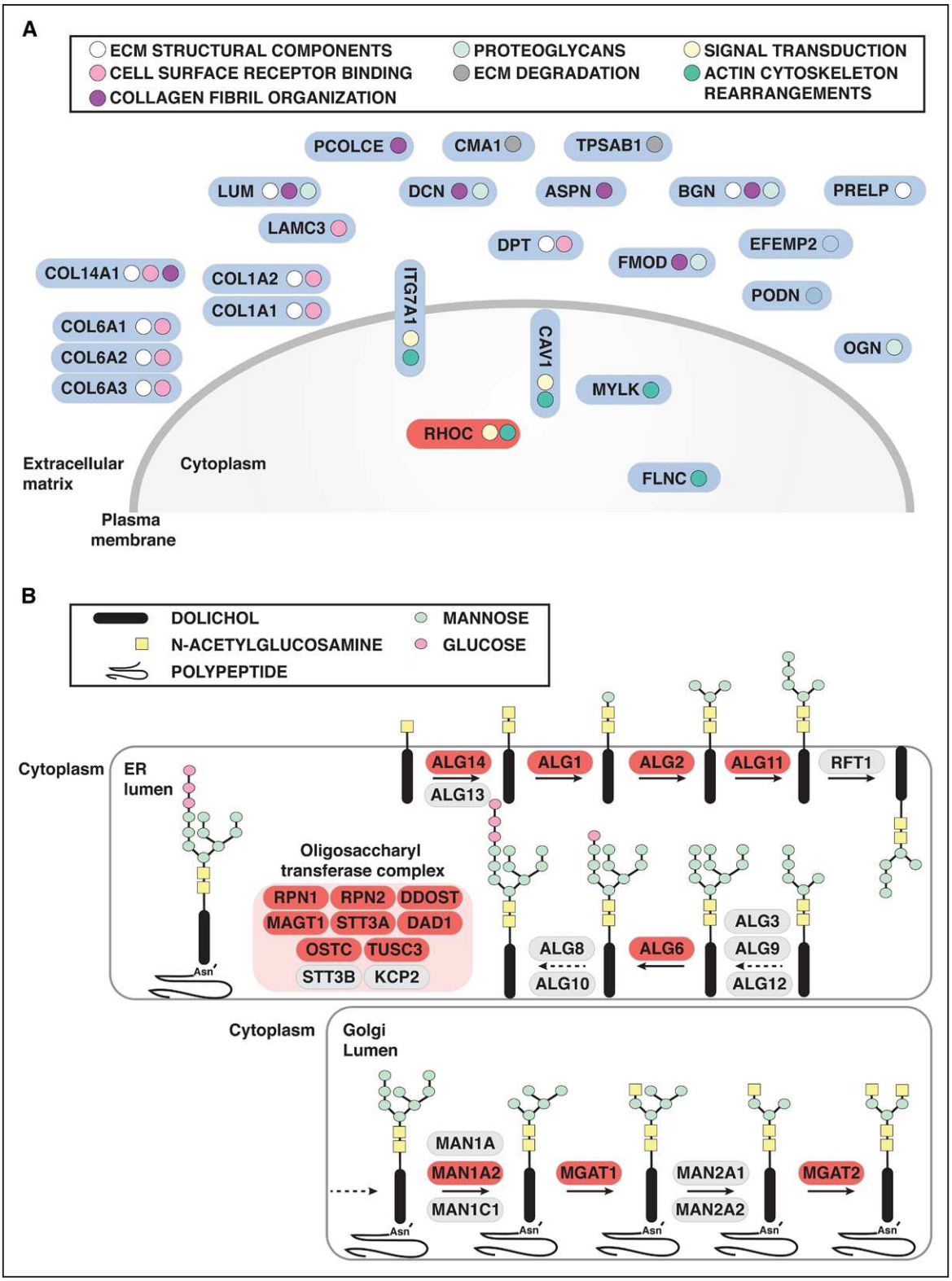

Figure 4. Proteomic analysis identifies novel aldosterone-producing adenoma (APA) associated pathways. **A**, APAs displayed changes in expression of various ECM (extracellular matrix) components, Integrin α-7, and proteins involved in actin cytoskeleton organization. **B**, APAs showed increased levels of multiple proteins involved in N-linked glycosylation. Proteins downregulated in APAs are boxed in blue while upregulated proteins are boxed in red. Proteins whose levels were not changed are boxed in gray.

an inhibitory neurotransmitter, is metabolized to succinate by the serial action of 2 enzymes, ABAT and ALDH5A1.[17] ABAT was significantly upregulated 1.58- to 4.20-fold in all 6 APAs compared with their corresponding controls (Table S5). ALDH5A1 levels were >1.5-fold higher in 3 adenomas and 1.28-fold higher in 1 adenoma. This observation

raises the possibility that APAs have reduced inhibitory GABAergic signaling.

Validation of the Proteomic Data

To validate the proteomics results, we examined 4 selected candidate proteins with differential expression in APA and nontumoral adrenal cortex by immunoblotting. We probed APA and Ctrl adrenal cortex protein lysates with antibodies against 3 members (RHOC, HX1, LSR) of the APA^{HIGH} cluster and 1 member (HSL) of the APA^{LOW} cluster. Immunoblot analysis confirmed the proteomic results for all proteins. RHOC, HX1, and LSR displayed overall higher expression in APAs, while HSL showed lower expression (Figure 5A and 5B). This validation by immunoblotting indicates the reliability of the presented proteomic data.

RHOC Regulates Expression of *CYP11B2* in Cultured Cells

The GTPase RHOC was upregulated in all tested adenomas (Table S5; Figure 5A and 5B). To understand the functional implications of this finding, we transiently transfected human adrenocortical NCI-H295R cells with a plasmid encoding *RHOC* (Figure 5C) and assessed proliferation and *CYP11B1* and *CYP11B2* mRNA levels. While *RHOC* overexpression did not affect cell proliferation (Figure 5D) or expression of *CYP11B1* (Figure 5E), it significantly increased expression of the gene encoding aldosterone synthase *CYP11B2* (Figure 5E). Thus, RHOC is a potential novel regulator of aldosterone synthesis. This observation also suggests that our approach allows identification of novel proteins involved in APA pathophysiology.

mTORC1 (Mammalian Target of Rapamycin Complex 1) Signaling Is Increased in APA

Phosphorylation regulates protein activity in numerous biological pathways. To assess which pathways are most affected by changes in phosphorylation in APA, we performed KEGG pathway enrichment analysis of phosphoproteins that had at least one significantly deregulated phosphorylation site in >50% of APA samples. This analysis identified mTOR (mammalian target of rapamycin) signaling to be the most deregulated pathway in adenomas (Figure 6A), and pointed toward deregulation of actin cytoskeleton rearrangements, mRNA metabolism, and protein chaperones (Figure 6A). mTOR is a serine/threonine kinase that forms 2 structurally and functionally distinct multiprotein complexes, mTORC1 (mTOR complex 1) and mTORC2 (mTOR complex 2).[18] Detailed analysis of proteins enriched under the KEGG term mTOR signaling suggested deregulation of mTORC1 rather than mTORC2 signaling. To confirm this, we probed APA and Ctrl adrenal cortex protein lysates for S6-pSer240/244 and Akt-pSer473, the well-established markers of mTORC1 and mTORC2 activity, respectively. S6-pSer240/244 expression was significantly increased in APAs compared with the respective

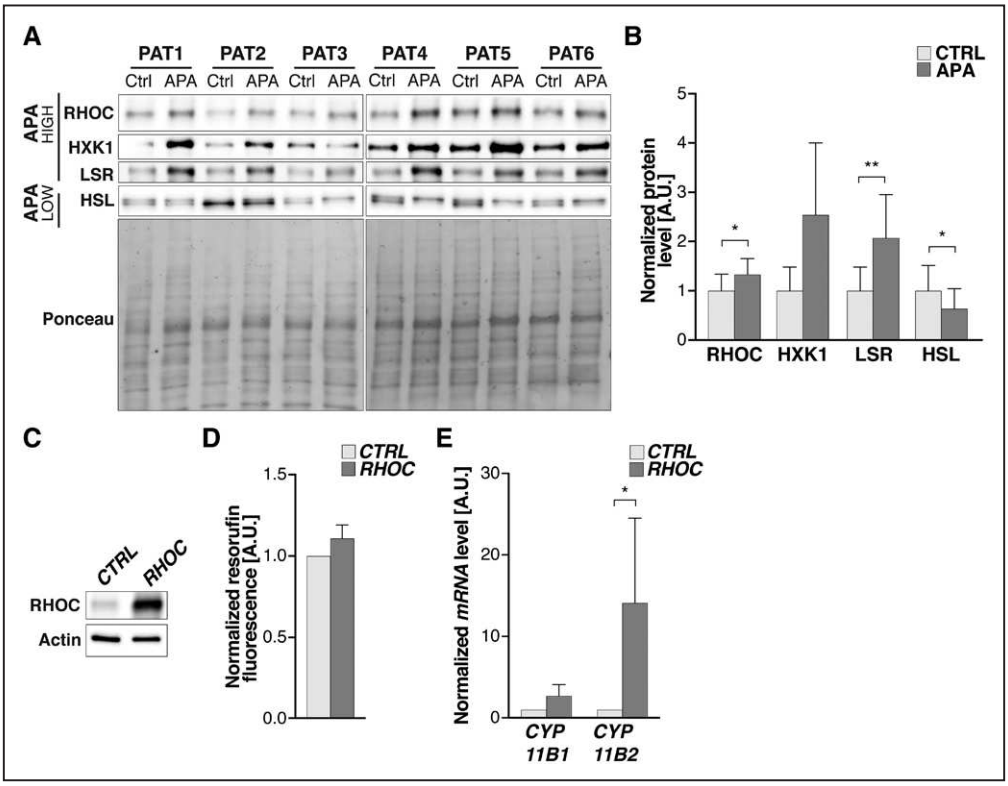

Figure 5. Validation of the proteomic data and identification of new proteins potentially involved in aldosterone-producing adenoma (APA) pathophysiology. **A**, Immunoblot validation of differential expression of selected proteins in APA and Ctrl adrenal cortex samples. **B**, Quantification of immunoblot signals presented in **A**. Data are shown as means±SD. **P*<0.05, ***P*<0.01 (paired *t* test). **C**, RHOC expression in NCI-H295R cells transiently transfected with *RHOC*-encoding vector. **D**, Proliferation assay of NCI-H295R cells transiently overexpressing *RHOC*. **E**, Expression of *CYP11B1* and *CYP11B2* in NCI-H295R cells transiently overexpressing *RHOC*. **C**–**E**, Control (Ctrl) cells were transfected with an empty plasmid. Data are presented as means±SD. **P*<0.05 (Wilcoxon matched-pairs signed rank test).

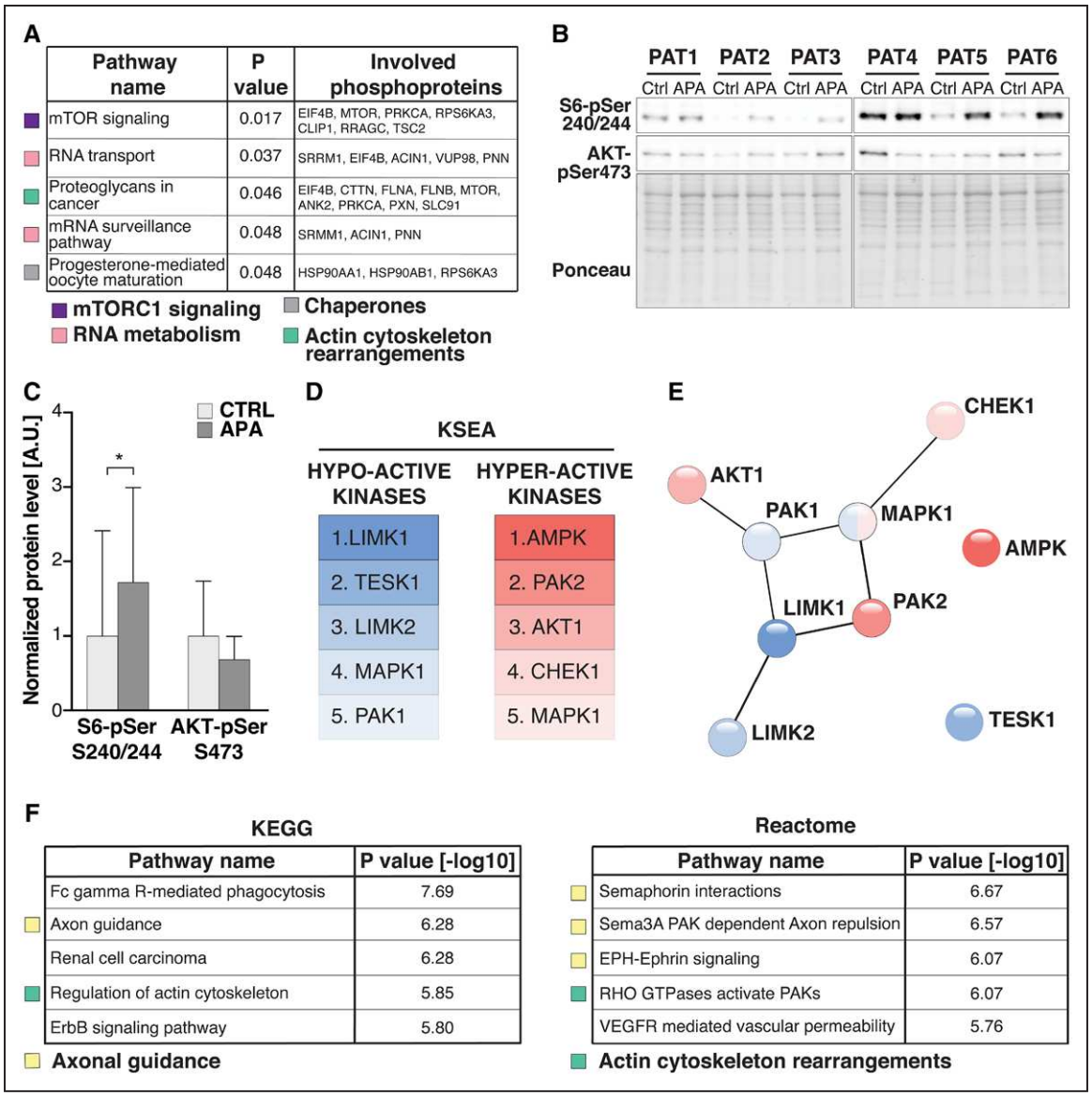

Figure 6. Phosphoproteomic analysis reveals signaling pathways and kinases deregulated in aldosterone-producing adenoma (APA). **A**, KEGG pathway enrichment analysis of phosphoproteins deregulated in at least 4 adenomas. **B**, Immunoblot evaluating mTORC1 (mammalian target of rapamycin complex 1) and mTORC2 (mTOR complex 2) signaling in APAs. **C**, Quantification of western blot signals presented in **B**. Data are shown as mean±SD, *P<0.05 (paired t test). **D**, Top hypoactive and hyperactive kinases in APA identified in kinase-substrate enrichment analysis (KSEA). **E**, String analysis and (**F**) KEGG/Reactome pathway enrichment analyses of the kinases identified in **D**.

controls, while Akt-pSer473 expression was not changed (Figure 6B and 6C). Thus, mTORC1 but not mTORC2 signaling seems to be increased in adenomas.

Kinase-Substrate Enrichment Analysis Reveals Kinases Deregulated in APA

To identify the top hypoactive and hyperactive kinases in APA, we performed kinase-substrate enrichment analysis. This method infers kinase activity by averaging the signals for multiple phosphosites targeted by a given kinase and is based on the analysis of curated substrate-kinase interactions deposited in public databases (Materials and Methods in the online-only Data Supplement). Of note, only 1 325 of 9 742 phosphosites quantified in this study have predicted kinases. This suggests that less characterized kinases might have an important role in APAs. Kinase-substrate enrichment analysis indicated decreased activity of LIMK1/2, TESK1, MAPK1, and PAK1 and increased activity of AMPK, PAK2, AKT1, CHEK1, and MAPK1 kinases in APA (Figure 6D). The presence of MAPK1 in both lists suggests that its activity was decreased toward certain targets (eg, CTTN) and increased toward others (eg, SLC9A1). String analysis revealed that most of the top deregulated kinases functionally or physically interact (Figure 6E), suggesting that they cooperate within the same signaling pathway(s). To identify such pathways, we performed KEGG and Reactome pathway enrichments of the top deregulated kinases. In both analyses, the terms corresponding to cytoskeleton remodeling and axonal guidance appeared among the top 5 identified pathways (Figure 6F). Thus, both proteomic and phosphoproteomic data indicate

that development of APA is accompanied by changes in cytoskeleton and possibly innervation.

Discussion

Here we present the first APA (phospho)proteome using deep quantitative mass spectrometry. Our results reveal potentially novel molecular mechanisms in APA pathophysiology. First, the finding that HSD3B2 and CYP21A2 are phosphorylated in APA suggests that the activity of steroidogenic enzymes is regulated by phosphorylation. This is further supported by the large-scale phosphoproteomic studies (http://www.phosphositeplus.org) indicating that the major steroidogenic enzymes (CYP11A1, CYP21A2, CYP17A1, AKR1C3, CYP11B1, and CYP11B2) are phosphorylated at multiple sites. Since C17–20 lyase activity of CYP17A1 is increased by phosphorylation,[12] it is possible that phosphorylation also modulates activity of other steroidogenic enzymes. Further studies on mechanisms and physiological relevance of steroidogenic enzyme phosphorylation are required.

Second, our observation that LSR is the only lipoprotein receptor upregulated in APA suggests that LSR mediates the uptake of cholesterol for steroidogenesis. However, LSR also has functions beyond lipoprotein uptake. LSR affects cell motility and invasion[19] and expression of genes encoding actin, tight junction proteins, and proteins affecting cancer progression.[19] Thus, increased expression of LSR in APA might not only provide cholesterol to support steroidogenesis but may also affect actin cytoskeleton dynamics and cell proliferation.

Third, our proteomic data suggest striking changes in ECM and actin cytoskeleton composition in APAs. It is well established, that binding of ECM proteins to their cognate receptors (integrins) on the cell surface initiates signaling cascades that control various cellular functions, including proliferation, differentiation, and migration.[14] Many of these functions are regulated via RHO GTPases and lead to changes in actin cytoskeleton structure.[15] It has also been shown that ECM composition has a profound effect on adrenocortical cell function. Signals from the extracellular environment are critical for fetal adrenal gland development, where they coordinate steroid hormone production and cell turnover.[20] Indeed, binding of fibronectin or collagens to cultured adrenocortical cells promotes steroidogenesis while laminins enhance proliferation.[21] ACTH (corticotropin) and Ang II (angiotensin II), the major regulators of steroidogenesis, exert their effect, at least in part, through promoting actin cytoskeleton rearrangements.[22,23] In addition, the RHOA-ROCK pathway, which acts downstream of ECM-integrin signaling, has been shown to differentially affect steroid hormone production by inhibiting glucocorticoid synthesis and promoting androgen production.[24] Here, we demonstrate that RHOC, another member of the RHO GTPases family, specifically regulates *CYP11B2* expression, and presumably aldosterone production, without affecting *CYP11B1* or cellular proliferation. Whether RHOC exerts this effect via regulating the actin cytoskeleton or by affecting other signaling pathways requires further research. Of note, mRNAs encoding various extracellular proteins are overexpressed in cortisol-producing adenomas,[25] further highlighting the role of ECM in adrenal gland pathophysiology.

Fourth, our observation that APAs display increased expression of various protein involved in protein N-glycosylation suggests a role for N-glycosylation in APA pathophysiology. Clients of this posttranslational modification include nearly all secreted and cell surface proteins, as well as many proteins residing in the ER, Golgi, or lysosomes. The modification promotes protein folding, stability, trafficking, localization and oligomerization, and affects cell-cell/ECM interactions, intracellular signaling, and cellular metabolism.[16,26] It is often deregulated in tumors, where it affects all steps of development and progression.[26] The role of N-glycosylation in the adrenal cortex has not been studied in detail, although the modification is prominent in adrenocortical cells and has been postulated to affect local synthesis and quality control of membrane proteins involved in cholesterol and steroid metabolism.[27] Key regulators of steroidogenesis, MC2R and AT1R (receptors for ACTH and Ang II, respectively), are N-glycosylated and their activity could be affected by glycosylation. Similarly, lipoprotein receptors (LDLR and SR-B1) and many ion channels affecting steroidogenesis (TASK1, TASK3, TREK1, Kv1.4, CACNA1D) are N-glycoproteins and their levels/activities could be affected by increased glycosylation. This might be particularly true for the ion channels. Disruption of TASK1 and TASK3 glycosylation lowers the number of cell surface channels, which leads to a reduced current flow.[28] Similarly, inhibiting N-glycosylation of Kv1.4 channel decreases protein stability, induces intracellular retention, and decreases cell surface protein levels.[29] Thus, enhanced N-glycosylation observed in APA may promote steroidogenesis via increasing cell surface expression and activity of relevant receptors and ion channels.

Fifth, our finding that APAs exhibit increased expression of 2 enzymes involved in GABA degradation suggests that reduced GABAergic signaling leads to APA. This hypothesis is further supported by the results of our phosphoproteomic analysis implicating kinases normally involved in axonal guidance. Activation of GABAergic signaling decreases steroidogenesis in the rat adrenal cortex in vivo,[30] and GABA receptors are expressed on the surface of human adrenal cortex cells.[31] Interestingly, adrenocortical cells themselves express GABA synthesis machinery.[31] Benzodiazepines, which enhance GABA action via $GABA_A$ receptor, inhibit steroid production by adrenocortical cells in vitro. Thus, it is likely that ABAT and ALDH5A1 overexpression in APA contributes to steroid hormone production by decreasing inhibitory GABAergic signaling. If this is the case, PA patients could benefit from treatment with GABA receptor agonists or ABAT inhibitors. Recently, it has been shown that ABAT controls the mitochondrial nucleoside salvage pathway, and its inhibition leads to decreased mitochondrial content.[32] Thus, increased ABAT expression in APA could also contribute to maintaining high mitochondrial count required to support enhanced steroidogenesis.

Last, our data indicate that mTORC1 signaling is the most deregulated pathway in APAs. This is consistent with previous histological studies showing that mTOR activity is commonly upregulated both in APA and BAH.[33] mTORC1 inhibition decreases the proliferation and steroidogenesis in

APA cells and immortalized adrenocortical cells.[33–35] Thus, increased mTORC1 signaling in APA could contribute to adenoma growth and increased steroidogenic output. Whether inhibition of mTORC1 provides patients with clinical benefit requires further studies.

APAs display different morphologies and gene expression patterns that correlate with genetic makeup.[36] The fact that we identified common pathways in different APAs suggests that such pathways are particularly important in APA pathophysiology. However, additional studies including more patients are necessary to fully characterize APA-associated changes, especially at the phosphoproteome level. We have observed clustering of KCNJ5^{mut} APA samples in 2 independent bioinformatic analyses, indicating a distinct protein expression pattern associated with the mutated KCNJ5 channel. Unfortunately, the low number of samples precluded a valid analysis of pathways deregulated specifically in response to the KCNJ5 mutation. In the same analyses, we also observed clustering of the control samples corresponding to the KCNJ5^{mut} APAs. While paracrine/systemic factors from the APAs are one potential reason for the observed clustering, environmental factors such as salt intake, age, different zonal composition, and background genetic factors may also play a role. Additional studies are necessary to correlate genotype with phenotype in APAs.

Perspectives

This first deep quantitative (phospho)proteomic study of APAs has shown that increased steroid hormone production in APA is accompanied by increased expression of steroidogenic enzymes and of the lipoprotein receptor LSR. It has demonstrated that the steroidogenic enzymes are phosphorylated, raising the possibility that these enzymes are regulated by protein kinases. Furthermore, our proteomic analysis has revealed that APAs have altered extracellular matrix composition, increased protein N-glycosylation and possibly reduced GABAergic signaling. Understanding the physiological and clinical relevance of these findings may translate into novel APA/PA therapeutic strategies.

Sources of Funding

This work was funded by the Novartis Foundation for Medical Biological Research (M. Hall and M.M. Swierczynska), Novartis/University of Basel Excellence Scholarship for Life Sciences (M.M. Swierczynska), the Swiss National Science Foundation (M. Hall), Goldschmidt-Jacobson Stiftung (M.J. Betz), Deutsche Forschungsgemeinschaft (F. Beuschlein) and Else Kroener-Fresenius Stiftung (M. Reincke).

Disclosures

None.

References

1. Buffolo F, Monticone S, Tetti M, Mulatero P. Primary aldosteronism in the primary care setting. *Curr Opin Endocrinol Diabetes Obes*. 2018;25:155–159. doi: 10.1097/MED.0000000000000408
2. Catena C, Colussi G, Sechi LA. Treatment of primary aldosteronism and organ protection. *Int J Endocrinol*. 2015;2015:597247. doi: 10.1155/2015/597247
3. Arlt W, Lang K, Sitch AJ, et al. Steroid metabolome analysis reveals prevalent glucocorticoid excess in primary aldosteronism. *JCI insight*. 2017;2:e93136. doi: 10.1172/jci.insight.93136
4. Beuschlein F, Reincke M, Arlt W. The impact of Connshing's syndrome - mild cortisol excess in primary aldosteronism drives diabetes risk. *J Hypertens*. 2017;35:2548. doi: 10.1097/HJH.0000000000001550
5. Aristizabal Prada ET, Castellano I, Susnik E, Yang Y, Meyer LS, Tetti M, Beuschlein F, Reincke M, Williams TA. Comparative genomics and transcriptome profiling in primary aldosteronism. *Int J Mol Sci*. 2018;19:E1124. doi: 10.3390/ijms19041124
6. Liu Y, Beyer A, Aebersold R. On the dependency of cellular protein levels on mRNA abundance. *Cell*. 2016;165:535–550. doi: 10.1016/j.cell.2016.03.014
7. Beuschlein F, Boulkroun S, Osswald A, et al. Somatic mutations in ATP1A1 and ATP2B3 lead to aldosterone-producing adenomas and secondary hypertension. *Nat Genet*. 2013;45:440, 444e1–444, 444e1. doi: 10.1038/ng.2550
8. Tan GC, Negro G, Pinggera A, et al. Aldosterone-producing adenomas: histopathology-genotype correlation and identification of a novel CACNA1D mutation. *Hypertension*. 2017;70:129–136. doi: 10.1161/HYPERTENSIONAHA.117.09057
9. Murakami M, Yoshimoto T, Nakabayashi K, Nakano Y, Fukaishi T, Tsuchiya K, Minami I, Bouchi R, Okamura K, Fujii Y, Hashimoto K, Hata KI, Kihara K, Ogawa Y. Molecular characteristics of the KCNJ5 mutated aldosterone-producing adenomas. *Endocr Relat Cancer*. 2017;24:531–541. doi: 10.1530/ERC-17-0117
10. Monticone S, Hattangady NG, Nishimoto K, et al. Effect of KCNJ5 mutations on gene expression in aldosterone-producing adenomas and adrenocortical cells. *J Clin Endocrinol Metab*. 2012;97:E1567–E1572. doi: 10.1210/jc.2011-3132
11. Arakane F, King SR, Du Y, Kallen CB, Walsh LP, Watari H, Stocco DM, Strauss JF III. Phosphorylation of steroidogenic acute regulatory protein (StAR) modulates its steroidogenic activity. *J Biol Chem*. 1997;272:32656–32662.
12. Zhang LH, Rodriguez H, Ohno S, Miller WL. Serine phosphorylation of human p450c17 increases 17,20-lyase activity: implications for adrenarche and the polycystic ovary syndrome. *Proc Natl Acad Sci USA*. 1995;92:10619–10623.
13. Miller WL, Auchus RJ. The molecular biology, biochemistry, and physiology of human steroidogenesis and its disorders. *Endocr Rev*. 2011;32:81–151. doi: 10.1210/er.2010-0013
14. Kim SH, Turnbull J, Guimond S. Extracellular matrix and cell signalling: the dynamic cooperation of integrin, proteoglycan and growth factor receptor. *J Endocrinol*. 2011;209:139–151. doi: 10.1530/JOE-10-0377
15. Jaffe AB, Hall A. Rho GTPases: biochemistry and biology. *Annu Rev Cell Dev Biol*. 2005;21:247–269. doi: 10.1146/annurev.cellbio.21.020604.150721
16. Freeze HH, Eklund EA, Ng BG, Patterson MC. Neurological aspects of human glycosylation disorders. *Annu Rev Neurosci*. 2015;38:105–125. doi: 10.1146/annurev-neuro-071714-034019
17. Pearl PL, Gibson KM. Clinical aspects of the disorders of GABA metabolism in children. *Curr Opin Neurol*. 2004;17:107–113.
18. Shimobayashi M, Hall MN. Making new contacts: the mTOR network in metabolism and signalling crosstalk. *Nat Rev Mol Cell Biol*. 2014;15:155–162. doi: 10.1038/nrm3757
19. Reaves DK, Fagan-Solis KD, Dunphy K, Oliver SD, Scott DW, Fleming JM. The role of lipolysis stimulated lipoprotein receptor in breast cancer and directing breast cancer cell behavior. *PLoS One*. 2014;9:e91747. doi: 10.1371/journal.pone.0091747
20. Chamoux E, Narcy A, Lehoux JG, Gallo-Payet N. Fibronectin, laminin, and collagen IV as modulators of cell behavior during adrenal gland development in the human fetus. *J Clin Endocrinol Metab*. 2002;87:1819–1828. doi: 10.1210/jcem.87.4.8359
21. Gallo-Payet N. 60 YEARS OF POMC: adrenal and extra-adrenal functions of ACTH. *J Mol Endocrinol*. 2016;56:T135–T156. doi: 10.1530/JME-15-0257
22. Otis M, Campbell S, Payet MD, Gallo-Payet N. In adrenal glomerulosa cells, angiotensin II inhibits proliferation by interfering with fibronectin-integrin signaling. *Endocrinology*. 2008;149:3435–3445. doi: 10.1210/en.2008-0282
23. Peverelli E, Catalano R, Giardino E, Treppiedi D, Morelli V, Ronchi CL, Vaczlavik A, Fusco N, Ferrero S, Bertherat J, Beuschlein F, Chiodini I, Arosio M, Spada A, Mantovani G. Cofilin is a cAMP effector in mediating actin cytoskeleton reorganization and steroidogenesis in mouse and human adrenocortical tumor cells. *Cancer Lett*. 2017;406:54–63. doi: 10.1016/j.canlet.2017.07.025
24. Sewer MB, Li D. Regulation of adrenocortical steroid hormone production by RhoA-diaphanous 1 signaling and the cytoskeleton. *Mol Cell Endocrinol*. 2013;371:79–86. doi: 10.1016/j.mce.2012.11.014

25. Wilmot Roussel H, Vezzosi D, Rizk-Rabin M, Barreau O, Ragazzon B, René-Corail F, de Reynies A, Bertherat J, Assié G. Identification of gene expression profiles associated with cortisol secretion in adrenocortical adenomas. *J Clin Endocrinol Metab*. 2013;98:E1109–E1121. doi: 10.1210/jc.2012-4237
26. Pinho SS, Reis CA. Glycosylation in cancer: mechanisms and clinical implications. *Nat Rev Cancer*. 2015;15:540–555. doi: 10.1038/nrc3982
27. Black VH, Sanjay A, van Leyen K, Lauring B, Kreibich G. Cholesterol and steroid synthesizing smooth endoplasmic reticulum of adrenocortical cells contains high levels of proteins associated with the translocation channel. *Endocrinology*. 2005;146:4234–4249. doi: 10.1210/en.2005-0372
28. Mant A, Williams S, Roncoroni L, Lowry E, Johnson D, O'Kelly I. N-glycosylation-dependent control of functional expression of background potassium channels K2P3.1 and K2P9.1. *J Biol Chem*. 2013;288:3251–3264. doi: 10.1074/jbc.M112.405167
29. Watanabe I, Zhu J, Recio-Pinto E, Thornhill WB. Glycosylation affects the protein stability and cell surface expression of Kv1.4 but Not Kv1.1 potassium channels. A pore region determinant dictates the effect of glycosylation on trafficking. *J Biol Chem*. 2004;279:8879–8885. doi: 10.1074/jbc.M309802200
30. Mishunina TM, Kononenko VY. Specific GABA binding in the adrenals and blood corticosteroid levels in stress in intact rats and rats with changes in the functional activity of the hypothalamo-pituitary-adrenal system. *Neurosci Behav Physiol*. 2002;32:109–112.
31. Metzeler K, Agoston A, Gratzl M. An Intrinsic gamma-aminobutyric acid (GABA)ergic system in the adrenal cortex: findings from human and rat adrenal glands and the NCI-H295R cell line. *Endocrinology*. 2004;145:2402–2411. doi: 10.1210/en.2003-1413
32. Besse A, Wu P, Bruni F, Donti T, Graham BH, Craigen WJ, McFarland R, Moretti P, Lalani S, Scott KL, Taylor RW, Bonnen PE. The GABA transaminase, ABAT, is essential for mitochondrial nucleoside metabolism. *Cell Metab*. 2015;21:417–427. doi: 10.1016/j.cmet.2015.02.008
33. Su H, Gu Y, Li F, Wang Q, Huang B, Jin X, Ning G, Sun F. The PI3K/AKT/mTOR signaling pathway is overactivated in primary aldosteronism. *PLoS One*. 2013;8:e62399. doi: 10.1371/journal.pone.0062399
34. De Martino MC, van Koetsveld PM, Feelders RA, Sprij-Mooij D, Waaijers M, Lamberts SW, de Herder WW, Colao A, Pivonello R, Hofland LJ. The role of mTOR inhibitors in the inhibition of growth and cortisol secretion in human adrenocortical carcinoma cells. *Endocr Relat Cancer*. 2012;19:351–364. doi: 10.1530/ERC-11-0270
35. Mariniello B, Rosato A, Zuccolotto G, Rubin B, Cicala MV, Finco I, Iacobone M, Frigo AC, Fassina A, Pezzani R, Mantero F. Combination of sorafenib and everolimus impacts therapeutically on adrenocortical tumor models. *Endocr Relat Cancer*. 2012;19:527–539. doi: 10.1530/ERC-11-0337
36. Seccia TM, Caroccia B, Gomez-Sanchez EP, Gomez-Sanchez CE, Rossi GP. The biology of normal zona glomerulosa and aldosterone-producing adenoma: pathological implications. *Endocr Rev*. 2018;39:1029–1056. doi: 201810.1210/er.2018-00060

Novelty and Significance

What Is New?

- Aldosterone-producing adenomas (APAs) increase steroidogenic output by upregulating steroidogenic enzymes (HSD3B2, CYP21A2, CYP11B2) and a receptor involved cholesterol uptake (LSR).
- HSD3B2 and CYP21A2 are phosphorylated, raising a possibility that activity of steroidogenic enzymes is commonly regulated by protein kinases.
- APAs have a distinct ECM (extracellular matrix) composition compared with the adjacent adrenal cortex, which affects ECM-cell surface interactions and actin cytoskeleton rearrangements.
- RHOC, a GTPase controlling reorganization of actin cytoskeleton in response to extracellular stimuli, is upregulated in adenomas and can influence expression of aldosterone synthase *CYP11B2* in vitro.
- APAs display higher levels of proteins involved in N-glycosylation and of enzymes controlling GABA degradation, suggesting that tumor development might be associated with increased protein glycosylation and decreased inhibitory GABAergic signaling.

What Is Relevant?

- Our study broadens the knowledge on APA pathophysiology and provides a rich resource for the future research on the molecular mechanisms and clinical management of primary aldosteronism.

Summary

We describe the first deep quantitative (phospho)proteome of aldosterone-producing adenomas. We identify novel proteins and pathways possibly involved in APA pathophysiology. Understanding clinical implications of these observations could lead to the development of novel treatments for primary aldosteronism.